Neurobiology of Disease

APOE Isoforms Control Pathogenic Subretinal Inflammation in Age-Related Macular Degeneration

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Contrary to Alzheimer's disease (AD), the *APOE2* allele increases and the *APOE4* allele reduces the risk to develop age-related macular degeneration (AMD) compared with the most common *APOE3* allele. The underlying mechanism for this association with AMD and the reason for the puzzling difference with AD are unknown. We previously demonstrated that pathogenic subretinal mononuclear phagocytes (MPs) accumulate in *Cx3cr1*-deficient mice due to the overexpression of APOE, interleukin-6, and CC chemokine ligand 2 (CCL2). We here show using targeted replacement mice expressing the human APOE isoforms (*TRE2*, *TRE3*, and *TRE4*) that MPs of *TRE2* mice express increased levels of APOE, interleukin-6, and CCL2 and develop subretinal MP accumulation, photoreceptor degeneration, and exaggerated choroidal neovascularization similar to AMD. Pharmacological inhibition of the cytokine induction inhibited the pathogenic subretinal inflammation. In the context of APOE-dependent subretinal inflammation in *Cx3cr1*$^{GFP/GFP}$ mice, the *APOE4* allele led to diminished APOE and CCL2 levels and protected *Cx3cr1*$^{GFP/GFP}$ mice against harmful subretinal MP accumulation observed in *Cx3cr1*$^{GFP/GFP}$*TRE3* mice. Our study shows that pathogenic subretinal inflammation is APOE isoform-dependent and provides the rationale for the previously unexplained implication of the APOE2 isoform as a risk factor and the APOE4 isoform as a protective factor in AMD pathogenesis.

Key words: apolipoprotein E; mononuclear phagocyte; neurodegeneration; neuroinflammation

Significance Statement

The understanding of how genetic predisposing factors, which play a major role in age-related macular degeneration (AMD), participate in its pathogenesis is an important clue to decipher the pathomechanism and develop efficient therapies. In this study, we used transgenic, targeted replacement mice that carry the three human APOE isoform-defining sequences at the mouse APOE chromosomal location and express the human APOE isoforms. Our study is the first to show how *APOE2* provokes and *APOE4* inhibits the cardinal AMD features, inflammation, degeneration, and exaggerated neovascularization. Our findings reflect the clinical association of the genetic predisposition that was recently confirmed in a major pooled analysis. They emphasize the role of APOE in inflammation and inflammation in AMD.

Introduction

In humans, the *APOE* gene has three common genetic variants (*APOE2*, *APOE3*, and *APOE4*), due to two polymorphisms rs7412 and rs429358 that are imbedded in a well-defined CpG island, and lead to two cysteine-arginine interchanges at residues 112 and 158 (Yu et al., 2013). The *APOE2* allele is associated with higher APOE concentrations in plasma, CSF, and brain tissue (Riddell et al., 2008; Bales et al., 2009) due to impaired clearance caused by APOE2's decreased affinity for the low-density lipoprotein receptor (Mahley and Rall, 2000). Its transcription can

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also be increased in certain cell types (astrocytes, neurons) due to the loss of CpG sites associated with *APOE3* and *APOE4* alleles (Yu et al., 2013). Compared with the *APOE3* allele, the *APOE4* allele is transcribed similarly in neurons and astrocytes (Yu et al., 2013), but its protein concentrations in plasma, CSF, and brain parenchyma are decreased (Riddell et al., 2008; Bales et al., 2009; Sullivan et al., 2011). The structural changes in the APOE4 protein also lead to diminished association with high-density lipoprotein (Dong and Weisgraber, 1996) and impaired reverse cholesterol transport (Heeren et al., 2004; Mahley et al., 2009).

APOE2 allele carriers are at increased risk for developing late age-related macular degeneration (AMD) [odds ratio (OR) = 1.83 for homozygote *APOE2* allele carriers] and are protected against Alzheimer's disease (AD), whereas the *APOE4* allele protects against AMD (OR = 0.72 per haplotype) and is a risk factor for AD compared with the most common *APOE3* allele (Mahley and Rall, 2000; McKay et al., 2011). This association was recently confirmed in a pooled study of >20,000 subjects (McKay et al., 2011). It is found for both clinical forms of late AMD: wet AMD, which is defined by choroidal neovascularization (CNV) and geographic atrophy, which is characterized by an extending lesion of both the retinal pigment epithelium (RPE) and photoreceptors. In AD, the *APOE4* allele is associated with greater β-amyloid burden, possibly due to reduced efficacy in clearance of β-amyloid via multiple pathways (Bales et al., 2009; Mahley et al., 2009). The mechanism underlying the associations of the APOE isoforms with AMD remains unexplained.

APOE is the main lipoprotein of the brain and the retina (Mahley and Rall, 2000; Anderson et al., 2001). It is strongly expressed in mononuclear phagocytes (MPs), such as macrophages and microglial cells (Peri and Nüsslein-Volhard, 2008; Levy et al., 2015), and plays a major role in macrophage lipid efflux and reverse cholesterol transport in conjunction with APOA-I (Mahley and Rall, 2000; Mahley et al., 2009). APOE and APOA-I can also induce interleukin-6 (IL-6) and CC chemokine ligand 2 (CCL2) in MPs in the absence of pathogen-derived ligands (Smoak et al., 2010; Levy et al., 2015).

We recently showed that subretinal MPs that accumulate in AMD strongly express APOE (Levy et al., 2015). The subretinal MPs of *Cx3cr1*$^{GFP/GFP}$ mice that develop subretinal inflammation and cardinal features of AMD (Combadière et al., 2007) express similar high levels of APOE (Levy et al., 2015), but also IL-6 (Levy et al., 2015) and CCL2 (Sennlaub et al., 2013). We showed that APOE-induced IL-6 release from MPs represses RPE immune suppression, prolongs subretinal MP survival, and promotes subretinal inflammation (Levy et al., 2015). Furthermore, we demonstrated that increased levels of CCL2 in *Cx3cr1*$^{GFP/GFP}$ mice recruit pathogenic inflammatory CCR2^{+} monocytes to the subretinal space (Sennlaub et al., 2013). In consequence, subretinal pathogenic MPs accumulate in *Cx3cr1*$^{GFP/GFP}$ mice due to increased MP recruitment and decreased MP elimination. *ApoE* deletion in *Cx3cr1*$^{GFP/GFP}$ mice prevented age- and stress-induced subretinal MP accumulation and reduced associated CNV (Levy et al., 2015).

We here investigated the influence of the APOE alleles and isoforms on subretinal inflammation and associated photoreceptor degeneration and choroidal neovascularization, major hallmarks of AMD.

Materials and Methods

Animals. Targeted replacement mice that express human APOE isoforms (*TRE2*, *TRE3*, and *TRE4*) were engineered as previously described (Sullivan et al., 1997) and provided as a generous gift by Dr. Patrick Sullivan, backcrossed with C57BL/6 mice to eliminate the Crb1^{rd8} contamination in the three strains and crossed to *Cx3cr1*$^{GFP/GFP}$mice (Charles River). Mice were housed in the animal facility under specific pathogen-free condition, in a 12/12 h light/dark (100–500 lux) cycle with water and normal diet food available *ad libitum*. All experimental protocols and procedures were approved by the local animal care ethics committee "Comité d'éthique en expérimentation animale Charles Darwin" (Ce5/2010/013; Ce5/2011/033; Ce5/2010/044). We used male mice for choroidal neovascularization experiments, whereas experiments on aged and light challenged mice were performed on mice of either sex, as we did not observe differences between the sexes in these conditions.

Light challenge and laser injury model. Two-month-old mice of either sex were adapted to darkness for 6 h, pupils dilated and exposed to constant green LED light (starting at 2 A.M., 4500 lux, JP Vezon equipements) for 4 d as previously described (Sennlaub et al., 2013). Laser coagulations were performed on male mice with a 532 nm ophthalmological laser mounted on an operating microscope (Vitra Laser, 532 nm, 450 mW, 50 ms, 250 μm) as previously described (Levy et al., 2015). Intravitreal injections of 2 μl of PBS, isotype control rat IgG1, and rat anti-mouse CD14 (BD Biosciences) were performed using glass capillaries (Eppendorf) and a microinjector. The 2 μl solution of the antibodies was injected at 50 μg/ml, corresponding to an intraocular concentration of 5 μg/ml assuming their dilution by ~1/10th in the intraocular volume.

Immunohistochemistry, CNV, and MP quantification and histology. RPE and retinal flatmounts were stained and quantified as previously described (Sennlaub et al., 2013) using polyclonal rabbit anti-IBA-1 (Wako) and rat anti-mouse CD102 (clone 3C4, BD Biosciences) appropriate secondary antibodies and counterstained with Hoechst if indicated. Preparations were observed with fluorescence microscope (DM5500, Leica). Histology of mice eyes and photoreceptor quantification were performed as previously described (Sennlaub et al., 2013).

Cell preparations and cell culture. In accordance with the Declaration of Helsinki, volunteers provided written and informed consent for the human monocyte expression studies, which were approved by the Centre national d'ophthalmologie des Quinze-Vingt hospital (Paris) ethics committees (no. 913572). Peripheral blood mononuclear cells were isolated from heparinized venous blood from healthy volunteer individuals by 1-step centrifugation on a Ficoll Paque layer (GE Healthcare) and sorted with EasySep Human Monocyte Enrichment Cocktail without CD16 Depletion Kit (Stem Cell Technology). Mouse peritoneal macrophages, bone marrow-derived monocytes, and photoreceptor outer segment (POS) isolation (all in serum-free X-Vivo 15 medium) were performed as previously described (Sennlaub et al., 2013). In specific experiments, cells were stimulated with the different recombinant human APOE isoforms (5 μg/ml, Leinco Technologies), recombinant human APOE3 90 min heat-denatured (5 μg/ml, Leinco Technologies), APOE3 (5 μg/ml) with LPS inhibitor polymyxin B (25 μg/ml, Calbiochem), rat anti-IgG isotype control (25 μg/ml, R&D Systems), rat anti-mouse CD14 (25 μg/ml, R&D Systems), mouse anti-IgG isotype control (25 μg/ml), mouse anti-human TLR2 (25 μg/ml, Invivogen), human IgA2 isotype control (25 μg/ml, Invivogen), human anti-human TLR4 (25 μg/ml, Invivogen), and POS prepared as previously described (Sennlaub et al., 2013).

Reverse transcription and real-time PCR and ELISA. IL-6, CCL2, and IL-1β RT-PCRs using Sybr Green (Invitrogen) and ELISAs using mouse or human IL-6 DuoSet (R&D Systems), mouse or human CCL2 Duoset (R&D Systems), and human APOE Pro kit (Mabtech) were performed as previously described (Sennlaub et al., 2013; Hu et al., 2015; Levy et al., 2015).

Statistical analysis. GraphPad Prism 5 (GraphPad Software) was used for data analysis and graphic representation. All values are reported as mean ± SEM. Statistical analysis was performed by one-way or two-way ANOVA followed by Dunnett's post test or Mann–Whitney test for comparison among means depending on the experimental design. The p values are indicated in the figure legends.

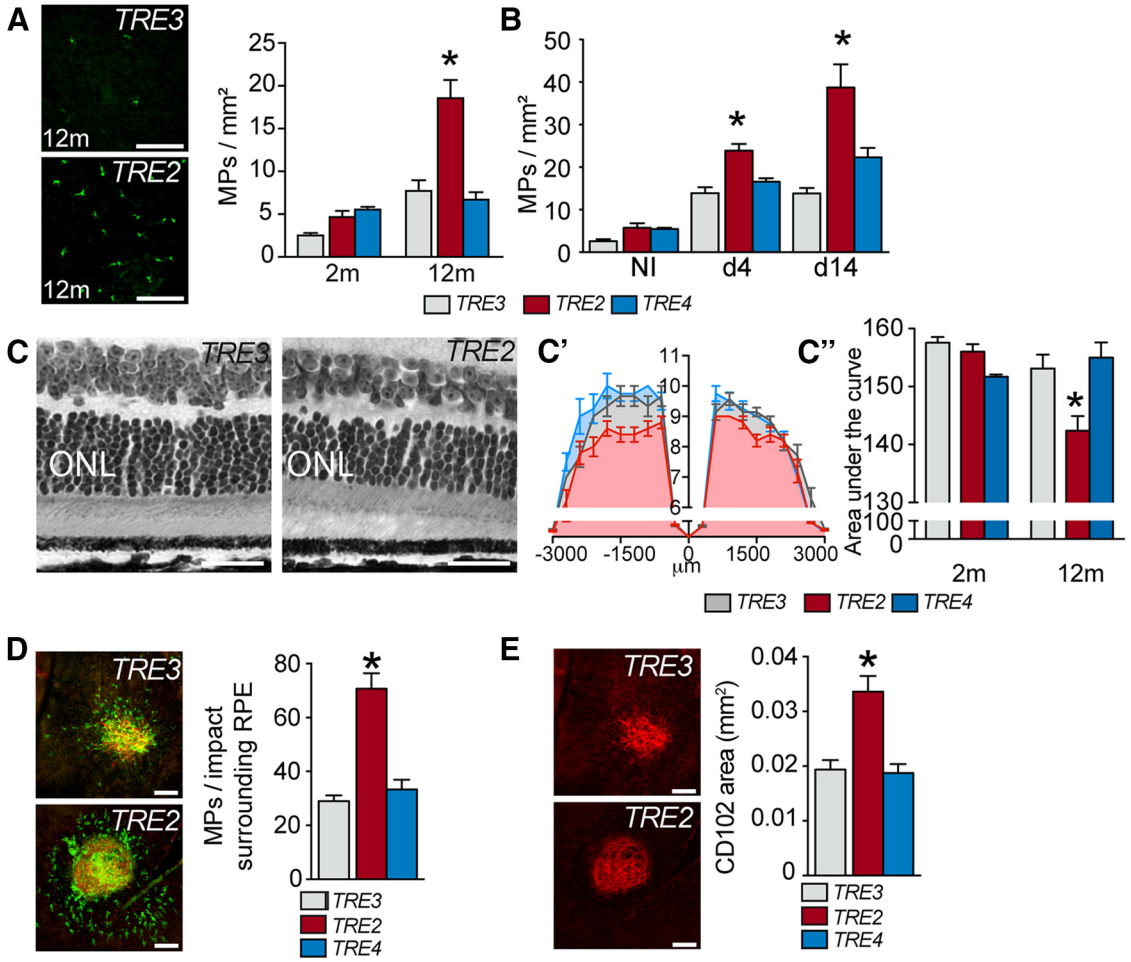

Figure 1. The *APOE2* allele leads to age- and stress-related subretinal MP accumulation, retinal degeneration, and exacerbated choroidal neovascularization. ***A***, Representative 12-month-old IBA-1-stained RPE flatmounts of *TRE3* and *TRE2* mice and quantification of subretinal IBA-1$^+$ MPs in 2- and 12-month-old mice of the indicated strains ($n = 9–20$/group). *$p < 0.0001$, 12 months versus *TRE3* control (ANOVA/Dunnett's multiple-comparison test). ***B***, Quantification of subretinal IBA-1$^+$ MPs after a 4 d light challenge followed by 10 d of normal light conditions (d14) of 2-month-old mice of the indicated strains ($n = 14–16$/group). *$p < 0.0001$, versus *TRE3* control at 14 d (ANOVA/Dunnett's multiple-comparison test). ***C***, Micrographs taken 1000 μm from the optic nerve of 12-month-old *TRE3* and *TRE2* mice. ONL, Outer nuclear layer. ***C'***, Photoreceptor nuclei rows at increasing distances (-3000 μm: inferior pole; 3000 μm: superior pole) from the optic nerve (0 μm) in 12-month-old mice. ***C''***, Quantification of the area under the curve of photoreceptor nuclei row counts of 2- and 12-month-old transgenic mouse strains ($n = 4–7$). *$p = 0.0102$, versus *TRE3* control at 12 months (ANOVA/Dunnett's multiple-comparison test). Mice were taken from several (≥ 3) independent cages for the quantifications. ***D***, CD102 (red) and IBA-1 (green) immunohistochemistry and quantification of subretinal IBA-1$^+$ MPs on the RPE counted at a distance of 0–500 μm to CD102$^+$ CNV 7 d after the laser injury of 2-month-old mice of the indicated strains ($n = 8–10$/group). *$p < 0.0001$ (one-way ANOVA/Dunnett's multiple-comparison test). ***E***, CD102 immunohistochemistry and quantification of CD102 area on RPE/choroidal flatmount from 2-month-old transgenic strains, 7 d after laser injury ($n = 8–10$/group). *$p < 0.0001$ (one-way ANOVA/Dunnett's multiple-comparison test). *TRE2–4*, Targeted replacement mice expressing human APOE isoforms Scale bars: ***A***, ***C–E***, 50 μm .

Results

The *APOE2* allele leads to age- and stress-related subretinal MP accumulation, retinal degeneration, and exacerbated choroidal neovascularization

The subretinal space, located between the RPE and the POS, does not contain significant numbers of MPs under normal conditions (Penfold et al., 2001; Gupta et al., 2003; Combadière et al., 2007; Levy et al., 2015). This is likely the result of physiologically low levels of chemoattractants along with strong immunosuppressive RPE signals that quickly eliminate infiltrating MPs (Sennlaub et al., 2013; Levy et al., 2015). We have previously shown that the lack of the tonic inhibitory CX3CL1/CX3CR1 signal, observed in *Cx3cr1*-deficient mice, is sufficient to induce pathogenic chronic subretinal MP accumulation as a consequence of increased recruitment and decreased elimination (Combadière et al., 2007; Sennlaub et al., 2013; Levy et al., 2015). We showed that this accumulation is dependent on the overexpression of APOE in *Cx3cr1*-deficient MPs (Levy et al., 2015). To evaluate a potential role of the human APOE isoforms in subretinal inflammation, we used targeted replacement mice expressing human isoforms (*TRE2*, *TRE3*, and *TRE4*) (Sullivan et al., 1997). We first backcrossed the strains with C57BL/6J mice to eliminate the *Crb1*^{rd8} contamination in the three strains, which can lead to AMD-like features (Mattapallil et al., 2012). The mice were raised under 12 h light/12 h dark cycles at 100–500 lux at the cage level, with no additional cover in the cage, the conditions that induce MP accumulation in *Cx3cr1*-deficient mice with age (Combadière et al., 2007). Quantification of subretinal IBA-1$^+$ MPs on retinal and RPE/choroidal flatmounts of 2- and 12-month-old *TRE2*, *TRE3*, and *TRE4* mice revealed that *TRE2* mice develop age-dependent subretinal MP accumulation compared with *TRE3* and *TRE4* mice (Fig. 1*A*). Similarly, *TRE2* mice accumulated significantly more subretinal MPs after a 4 d light challenge, and the MPs continued to accumulate after return for 10 additional days in normal light conditions (Fig. 1*B*; the intensity of our light challenge model

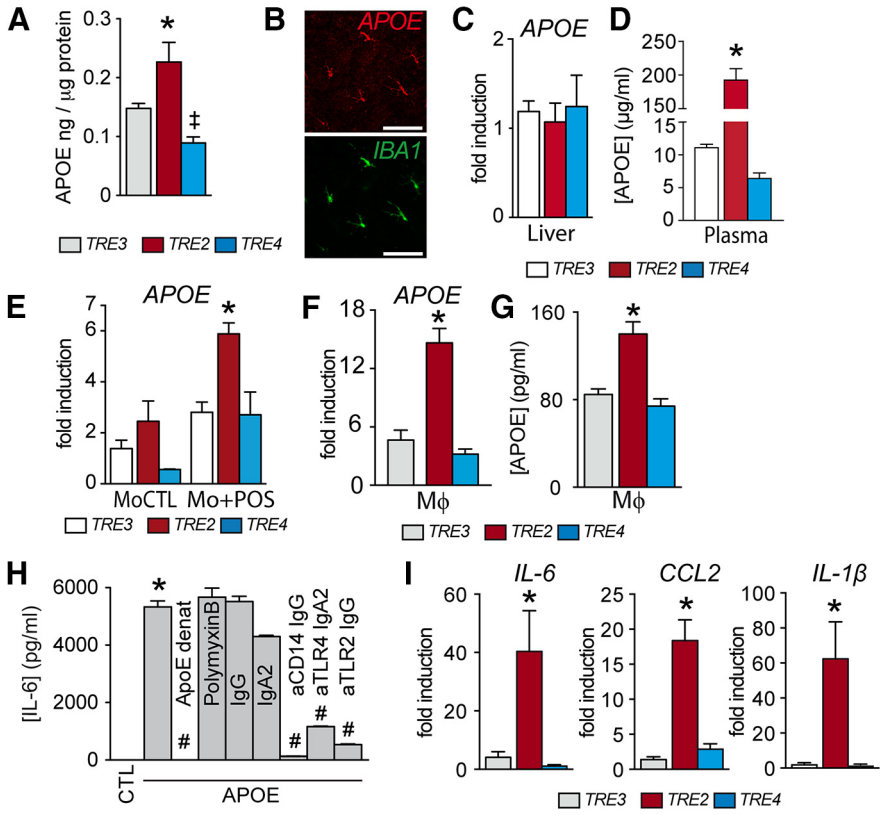

Figure 2. The *APOE2* allele increases APOE levels in the eye and APOE transcription and IIRC activation in MPs. ***A***, APOE ELISA of homogenates of PBS-perfused posterior segments of 12-month-old *TRE2*, *TRE3*, and *TRE4* mice ($n = 5$ or 6/group). $^{*}p = 0.0027$, versus *TRE3* control (one-way ANOVA/Dunnett's multiple-comparison test). $‡p = 0.0027$ (one-way ANOVA/Dunnett's multiple-comparison test). ***B***, Immunohistochemistry of APOE (red, top) and IBA-1 (green, bottom) of the subretinal side of a retinal flatmount from a 12-month-old *TRE2* mouse (representative of three independent experiments; experiments omitting the primary antibody immunostaining served as negative controls). ***C***, Quantitative RT-PCR of *ApoE* mRNA normalized with S26 mRNA of liver extracts from transgenic replacement mice expressing human APOE isoforms (*TRE2*, *TRE3*, and *TRE4* mice, $n = 3$). ***D***, ELISA quantification of APOE plasma concentrations in transgenic replacement mice expressing human APOE isoforms (*TRE2*, *TRE3*, and *TRE4* mice, $n = 3$). $^{*}p < 0.0001$, versus *TRE3* control (ANOVA/ Dunnett's multiple-comparison test). ***E***, Quantitative RT-PCR of *ApoE* mRNA normalized with *S26* mRNA of bone marrow-derived monocytes from transgenic replacement mice expressing human APOE isoforms (*TRE2*, *TRE3*, and *TRE4* mice) cultured for 3 d with or without porcine photoreceptor outer segments to simulate subretinal monocyte to macrophage differentiation ($n = 6$/group). $^{*}p = 0.0033$, Mo+POS versus *TRE3* control (ANOVA/ Dunnett's multiple-comparison test). ***F***, Quantitative RT-PCR of *ApoE* mRNA normalized with *S26* mRNA of peritoneal Mϕs from *TRE* mice cultured for 24 h ($n = 6$). $^{*}p < 0.0001$, versus *TRE3* control (ANOVA/Dunnett's multiple-comparison test). ***G***, APOE-ELISA of supernatants of peritoneal Mϕs from *TRE* mice cultured for 24 h ($n = 6$). $^{*}p < 0.0001$, versus *TRE3* control (ANOVA/Dunnett's multiple-comparison test). ***H***, Human IL-6 ELISA of supernatants from human monocytes incubated for 24 h in control medium, APOE3 (5 µg/ml), heat-denaturated APOE3 (dAPOE3, 5 µg/ml), APOE3 (5 µg/ml), and polymyxin B (25 µg/ml), APOE3 (5 µg/ml), and rat IgG1 isotype, or human IgA2 isotype control, or mouse IgG1 isotype control, or rat anti-CD14 IgG1 antibody, or human anti-TLR4 IgGA2 antibody, or mouse anti-TLR2 IgG1 antibody (all antibodies at 25 µg/ml) ($n = 4$–6/group). $^{*}p < 0.0001$, APOE3 versus CTL (one-way ANOVA/Bonferroni multiple-comparison test). $^{\#}p < 0.0001$, dAPOE3 versus APOE3 (one-way ANOVA/Bonferroni multiple-comparison test). $^{\#}p < 0.0001$, APOE3 IgG versus APOE3 aCD14 Ab (one-way ANOVA/Bonferroni multiple-comparison test). $^{\#}p < 0.0001$, APOE3 IgG versus APOE3 aTLR4 Ab (one-way ANOVA/Bonferroni multiple-comparison test). $^{\#}p < 0.0001$, APOE3 IgA versus APOE3 aTLR2 Ab (one-way ANOVA/Bonferroni multiple-comparison test). ***I***, Quantitative RT-PCR of Il-6, Ccl-2, and Il1β mRNA normalized with S26 mRNA ($n = 6$) of peritoneal Mφs from *TRE* mice cultured for 24 h. $^{*}p = 0.0069$, versus *TRE3* control IL-6 (ANOVA/Dunnett's multiple-comparison test). $^{*}p < 0.0001$, versus *TRE3* control CCL2 (ANOVA/ Dunnett's multiple-comparison test). $^{*}p = 0.0097$, versus *TRE3* control IL-1β (ANOVA/Dunnett's multiple-comparison test). *TRE2–4*, Targeted replacement mice expressing human APOE isoforms. Scale bar: ***B***, 50 µm.

used herein was calibrated to induce subretinal inflammation in inflammation-prone *Cx3cr1*$^{GFP/GFP}$ mice but not in *WT* mice) (Sennlaub et al., 2013).

We also observed a thinning of the outer nuclear layer that contains the photoreceptor nuclei on histological retinal sections from 12-month-old *TRE2* mice compared with *TRE3* mice (Fig. 1*C*; micrographs taken at equal distance from the optic nerve). Photoreceptor nuclei row counts (Fig. 1*C*′) and calculation of the area under the curve (Fig. 1*C*″) revealed that the age-related accumulation of subretinal MPs in *TRE2* mice is associated with significant photoreceptor cell loss compared with *TRE3* and *TRE4* mice.

In addition, in laser-induced CNV, subretinal IBA-1^{+} MPs (green staining, counted on the RPE at a distance of 0–500 µm to CD102^{+} CNVs, red staining) were significantly more numerous in *TRE2* mice 7 d after a laser impact (Fig. 1*D*) and had developed significantly greater CNV lesions (Fig. 1*E*) compared with the other strains.

Together, our data demonstrate that *TRE2* mice, expressing the *APOE2* AMD risk allele, develop age-related subretinal inflammation and photoreceptor degeneration and exaggerated inflammation and CNV after laser injury similar to late AMD.

The *APOE2* allele increases *APOE* levels in the eye and *APOE* transcription and innate immunity receptor cluster (IIRC) activation in MPs

We previously showed that the levels of soluble APOE are elevated in adult *TRE2* mouse brains and diminished in *TRE4* brains compared with *TRE3* mice in a model of AD (Bales et al., 2009). Similarly, ELISA of APOE levels of homogenates of PBS-perfused posterior segments (retina and RPE/choroid plexus) of 12-month-old mice revealed significantly higher levels of APOE in *TRE2* mice compared with *TRE3* and *TRE4* mice (Fig. 2*A*). Furthermore, immunohistochemical localization of APOE on retinal flatmounts of *TRE2* mice (Fig. 2*B*, red staining) revealed strong APOE expression in subretinal IBA-1^{+} MPs (Fig. 2*B*, green staining).

The polymorphism rs7412 that defines the APOE2 isoform also leads to the loss of a CpG site in the *APOE2* allele that has been shown to moderately, but significantly, increase *APOE* transcription in brain astrocytes, but not in hepatocytes (Yu et al., 2013). Our data confirm that APOE transcription in hepatocytes does not differ between genotypes (Yu et al., 2013) (Fig. 2*C*, RT-PCR) and that APOE concentrations in the blood were significantly increased in *TRE2* mice (Fig. 2*D*, blood plasma ELISA), shown to be due to its decreased clearance rate (Mahley and Rall, 2000). However, bone marrow-derived monocytes (cultured with POS for 3 d to mimic subretinal macrophage differentiation) (Fig. 2*E*, RT-PCR) and peritoneal macrophages (Fig. 2*F*, RT-PCR) from *TRE2* mice transcribed significantly higher levels of *ApoE* mRNA compared with MPs of the other mouse strains. Ac-

cordingly, the APOE secretion of *TRE2* mice macrophages was robustly increased (Fig. 2*G*, ELISA of supernatant) compared with the other groups.

APOE and APOA-I have been shown to activate the TLR2-TLR4 CD14-dependent IIRC in mouse peritoneal macrophages in the absence of pathogen-derived ligands and to induce inflammatory cytokines, such as IL-6 (Smoak et al., 2010; Levy et al., 2015), but also CCL2 (shown for APOA-I) (Smoak et al., 2010). We here show that human blood-derived CD14$^+$ monocytes significantly secrete IL-6 after 24 h of recombinant lipid-free APOE3 stimulation (Fig. 2*H*) similar to mouse macrophages. Ninety minute heat denaturation completely abolished the induction, whereas the LPS inhibitor polymyxin B did not, confirming that LPS contamination of APOE3 is not accountable for the effect, as shown for APOA-I using multiple approaches (Smoak et al., 2010). This induction was due to the activation of the TLR2-TLR4 CD14-dependent IIRC, as neutralizing antibodies to CD14, TLR2, and TLR4 inhibited this effect, compared with control antibodies (Fig. 2*H*). Accordingly, peritoneal macrophages from *TRE2* mice that express increased amounts of APOE (Fig. 2*C*,*D*) also transcribed significantly more IL-6, CCL2, and IL-1β compared with macrophages of the other isoforms (Fig. 2*I*).

Together, our results show that the *APOE2* allele increases APOE levels in the tissue and *APOE* expression in MPs. We confirm that APOE activates the IIRC and show that the excessive APOE expression in macrophages from *TRE2* mice is associated with increased production of inflammatory cytokines *in vitro*.

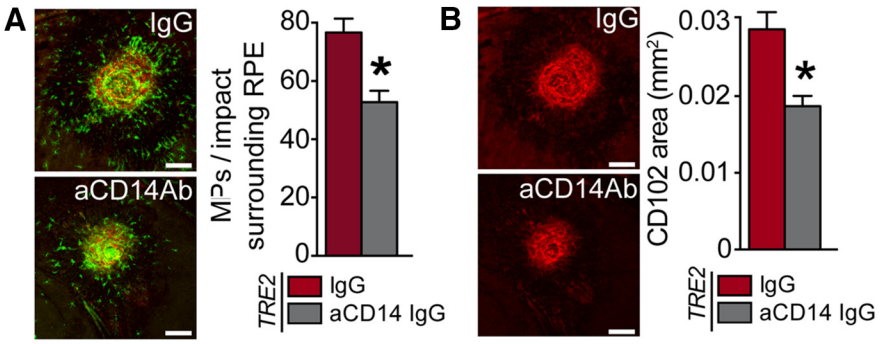

Figure 3. IIRC inhibition reduces subretinal MP accumulation and choroidal neovascularization in *TRE2* mice *in vivo*. ***A***, ***B***, Seven day laser-injured IBA-1 (green) and CD102 (red) stained RPE flatmounts of control IgG and anti-CD14-treated *TRE2* mice. Quantification of subretinal IBA-1$^+$ MPs/impact localized on the lesion surrounding RPE (***A***) and quantification of CD102$^+$ CNV area (***B***) of *TRE2* mice treated with control IgG or CD14-blocking antibodies (intraocular antibody concentration 5 μg/ml; $n = 12$/group). ***A***, $^*p = 0.0012$ (Mann–Whitney *t* test). ***B***, $^*p = 0.0009$ (Mann–Whitney *t* test). *TRE2–4*, Targeted replacement mice expressing human APOE isoforms; Scale bars: ***A*** and ***B***, 50 μm.

IIRC inhibition reduces subretinal MP accumulation and choroidal neovascularization in *TRE2* mice *in vivo*

To evaluate whether increased IIRC activation is implicated in subretinal MP accumulation observed in *TRE2* mice *in vivo*, we inhibited the IIRC by an intravitreal injection of a CD14-neutralizing antibody in the laser-induced CNV model (Figure 3). The antibody, which blocks APOE-dependent cytokine induction (Levy et al., 2015), inhibited subretinal MP accumulation around the laser injury, quantified on IBA1-stained RPE/choroidal flatmounts (Fig. 3*A*) and CD102$^+$ CNV formation (Fig. 3*B*) at day 7 after laser injury of *TRE2* mice compared with control IgG.

These results confirm that the CD14-dependent inflammatory cytokine induction participates in subretinal MP accumulation in *TRE2* mice *in vivo*, similar to *Cx3cr1*$^{GFP/GFP}$ mice (Levy et al., 2015).

The *APOE4* allele protects APOE-overexpressing *Cx3cr1*$^{GFP/GFP}$ mice from subretinal MP accumulation, retinal degeneration, and exacerbated choroidal neovascularization

Cx3cr1-deficient mice lack the tonic inhibitory signal of neuronal CX3CL1 and develop subretinal MP accumulation and concomitant photoreceptor degeneration with age when raised in cyclic light at 100–500 lux (Combadière et al., 2007; Chinnery et al., 2012; Sennlaub et al., 2013; Hu et al., 2015; Levy et al., 2015). The accumulation can be prevented by raising the animals in darkness (Combadière et al., 2007) or in dim light conditions (Luhmann et al., 2013) and be accelerated by a light challenge (Sennlaub et al., 2013; Hu et al., 2015; Levy et al., 2015) (for more details, see Sennlaub et al., 2013, mini review in supplemental data). Although these features do not mimic all the aspects of AMD (Drusen formation and RPE atrophy), they do model subretinal inflammation and associated photoreceptor degeneration, two hallmarks of AMD (Gupta et al., 2003). *Cx3cr1* deletion also increases subretinal MP accumulation in diabetes (Kezic et al., 2013), in a paraquat-induced retinopathy model (Chen et al., 2013), and in a retinitis pigmentosa model (Peng et al., 2014). We previously demonstrated that pathogenic MPs accumulate in *Cx3cr1*-deficient mice due to the overexpression of APOE, IL-6, and CCL2 (Sennlaub et al., 2013; Levy et al., 2015). To evaluate a possible influence of the APOE4 isoform in a model of pathological subretinal inflammation, we crossed *TRE3* and *TRE4* mice to *Cx3cr1*$^{GFP/GFP}$ mice (Levy et al., 2015).

Quantification of subretinal IBA-1$^+$ MPs on retinal and RPE/choroidal flatmounts of 2- and 12-month-old *Cx3cr1*$^{GFP/GFP}$*TRE3* mice and *Cx3cr1*$^{GFP/GFP}$*TRE4* mice revealed that the age-dependent subretinal MP accumulation observed in *Cx3cr1*$^{GFP/GFP}$*TRE3* mice was prevented in *Cx3cr1*$^{GFP/GFP}$*TRE4* mice (Fig. 4*A*). A 4 d light challenge led to similar initial subretinal MP accumulation, but the increase of MPs after return to normal light conditions was significantly blunted in *Cx3cr1*$^{GFP/GFP}$*TRE4* mice compared with *Cx3cr1*$^{GFP/GFP}$*TRE3* mice (Fig. 4*B*).

Furthermore, micrographs of histological sections of 12-month-old mice revealed a thicker outer nuclear layer in *Cx3cr1*$^{GFP/GFP}$*TRE4* mice compared with the thinned *Cx3cr1*$^{GFP/GFP}$*TRE3* mice (Fig. 4*C*). Photoreceptor nuclei row counts (Fig. 4*C*′) and calculation of the area under the curve (Fig. 4*C*″) show that the inhibition of the age-related accumulation of subretinal MPs in *Cx3cr1*$^{GFP/GFP}$*TRE4* mice compared with *Cx3cr1*$^{GFP/GFP}$*TRE3* mice significantly inhibited the associated photoreceptor cell loss. The age-related subretinal MP accumulation and photoreceptor degeneration observed in *Cx3cr1*$^{GFP/GFP}$*TRE3* mice are significantly increased compared with *TRE3* mice presented in Figure 1, similar to *Cx3cr1*$^{GFP/GFP}$ mice expressing mouse APOE (Sennlaub et al., 2013) (MP/mm^2 of 12-month-old mice: *TRE3* mice: 7.315 ± 1.72 SEM; *Cx3cr1*$^{GFP/GFP}$*TRE3* mice: 16.6 ± 2.22 SEM; area under the curve: *TRE3* mice: 148.3 ± 1.52 SEM; *Cx3cr1*$^{GFP/GFP}$*TRE3* mice: 137.3 ± 2.12 SEM).

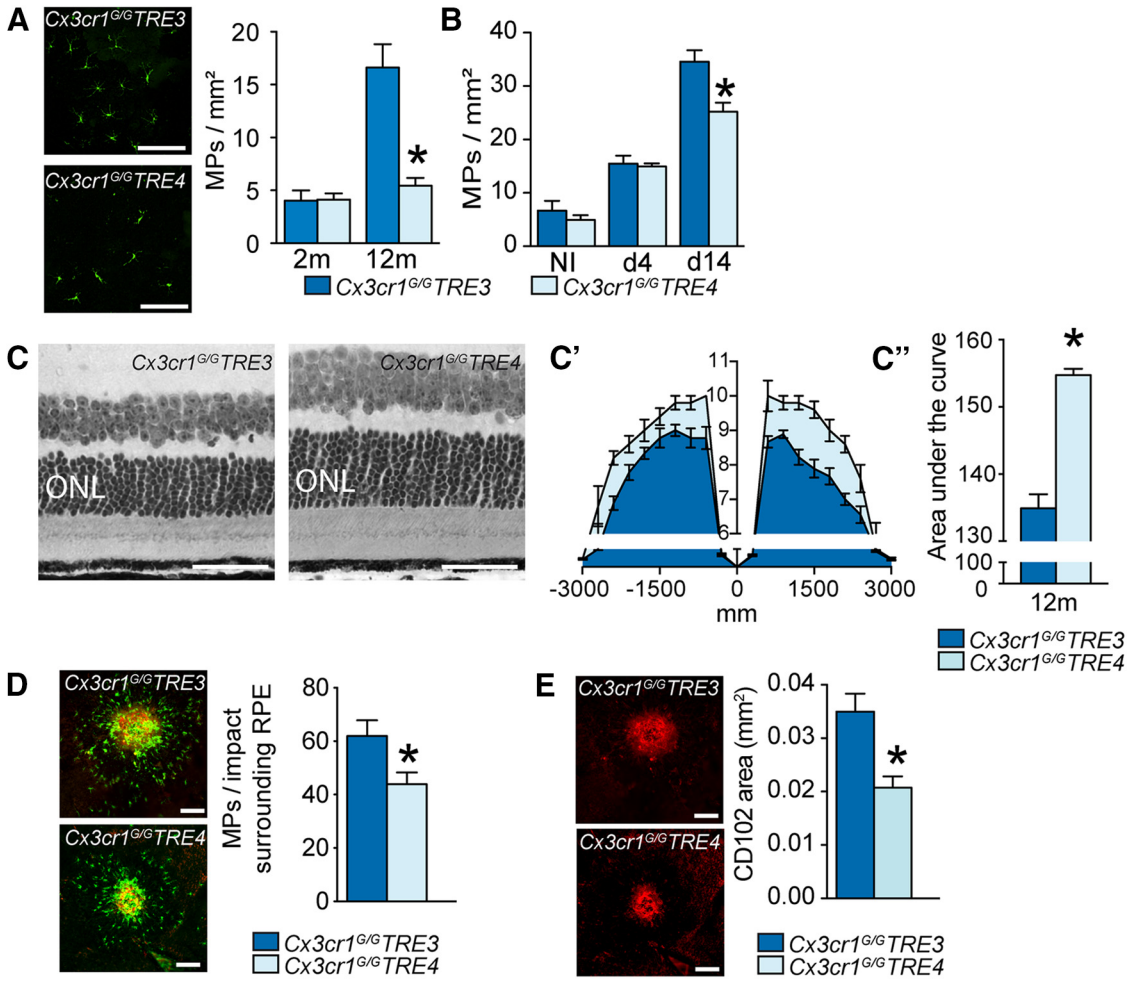

Figure 4. The *APOE4* allele protects APOE-overexpressing *Cx3cr1*$^{GFP/GFP}$ mice from subretinal MP accumulation, retinal degeneration, and exacerbated choroidal neovascularization. ***A***, Representative 12-month-old IBA-1-stained RPE flatmounts of *Cx3cr1*$^{GFP/GFP}$*TRE3* mice and *Cx3cr1*$^{GFP/GFP}$*TRE4* mice and quantification of subretinal IBA-1$^+$ MPs in 2- and 12-month-old mice of the indicated strains ($n = 8–13$/group). *$p = 0.0034$ (Mann–Whitney *t* test at 12 months). ***B***, Quantification of subretinal IBA-1$^+$ MPs after a 4 d light challenge followed by 10 d of normal light conditions (d14) of 2-month-old mice of the indicated strains ($n = 18$/group). *$p = 0.0036$ (Mann–Whitney *t* test at d14). ***C***, Micrographs, taken 1000 μm from the optic nerve of 12-month-old *Cx3cr1*$^{GFP/GFP}$*TRE3* mice and *Cx3cr1*$^{GFP/GFP}$*TRE4* mice. ONL, Outer nuclear layer. ***C′***, Photoreceptor nuclei rows at increasing distances (−3000 μm: inferior pole; 3000 μm: superior pole) from the optic nerve (0 μm) in 12-month-old mice. ***C″***, Quantification of the area under the curve of photoreceptor nuclei row counts of 12-month-old transgenic mouse strains ($n = 5–9$). *$p = 0.0032$ (Mann–Whitney *t* test). Mice were taken from several (≥3) independent cages for the quantifications. ***D***, CD102 (red) and IBA-1 (green) immunohistochemistry and quantification of subretinal IBA-1$^+$ MPs on the RPE counted at a distance of 0–500 μm to CD102$^+$ CNV 14 d after the laser injury of 2-month-old mice of the indicated strains ($n = 7$/group). *$p = 0.0182$ (Mann–Whitney *t* test). ***E***, CD102 immunohistochemistry and quantification of CD102 area on RPE/choroidal flatmount from 2-month-old transgenic strains, 14 d after laser injury ($n = 7$/group). *$p = 0.0034$ (Mann–Whitney *t* test). Scale bars: ***A***, ***C–E***, 50 μm.

Moreover, laser-induced subretinal IBA-1$^+$ MPs in 2-month-old mice (green staining) adjacent to CD102$^+$ CNVs (red staining) was again significantly inhibited in *Cx3cr1*$^{GFP/GFP}$*TRE4* mice compared with *Cx3cr1*$^{GFP/GFP}$*TRE3* mice (Fig. 4*D*) and had developed significantly greater CNV lesions at 14 d after laser injury (Fig. 4*E*) compared with the other strains.

In summary, our data demonstrate that the *APOE4* allele, which is protective for AMD, inhibits subretinal inflammation and concomitant degeneration and CNV in Cx3cr1 deficiency compared with *APOE3*.

The *APOE4* allele decreases ocular APOE levels in *Cx3cr1*$^{GFP/GFP}$ mice and activates the IIRC inefficiently

To investigate whether the *APOE3* and *APOE4* allele influences the APOE level in the eyes of *Cx3cr1*$^{GFP/GFP}$*TRE* mice, we analyzed APOE levels of homogenates of PBS-perfused posterior segments (retina and RPE/choroid plexus) of 12-month-old mice. APOE levels were significantly lower in homogenates of 12-month-old *Cx3cr1*$^{GFP/GFP}$*TRE4* mice compared with *Cx3cr1*$^{GFP/GFP}$*TRE3* mice (Fig. 5*A*), similar to APOE levels in the eyes of *TRE* mice (see above) and brains of PDAPP mice expressing human APOE isoforms (Bales et al., 2009).

APOA-I and APOE likely activate the IIRC by modifying the cholesterol content of the lipid rafts in which they are located (Smoak et al., 2010). As the APOE4 isoform has an impaired capacity to promote cholesterol efflux and transport (Heeren et al., 2004; Mahley et al., 2009), we next tested its ability to activate the IIRC of blood-derived human monocytes in culture. Interestingly, stimulation of monocytes for 24 h by recombinant APOE4 induced significantly less IL-6 and CCL2 secretion compared with the induction of the cytokines by equimolar concentrations of APOE3 (Fig. 5*B*).

APOE transcription (Fig. 5*C*, RT-PCR) and APOE secretion (Fig. 5*D*, ELISA of supernatant) in peritoneal macrophages from *Cx3cr1*$^{GFP/GFP}$*TRE3* mice and *Cx3cr1*$^{GFP/GFP}$*TRE4* mice were comparable, similar to previous reports from astrocytes: the

APOE4 allele did not diminish APOE production (Yu et al., 2013). However, in accordance with a decreased ability to activate the IIRC, peritoneal macrophages from *Cx3cr1*$^{GFP/GFP}$*TRE4* mice transcribed significantly less *CCL2* compared with macrophages from *Cx3cr1*$^{GFP/GFP}$ *TRE3* mice, whereas the IL-6 transcription was variable, but not significantly different (Fig. 5*E*).

Together, our results show that the *APOE4* allele leads to decreased APOE tissue levels and to a reduced capacity to activate the IIRC and induce CCL2 in MPs.

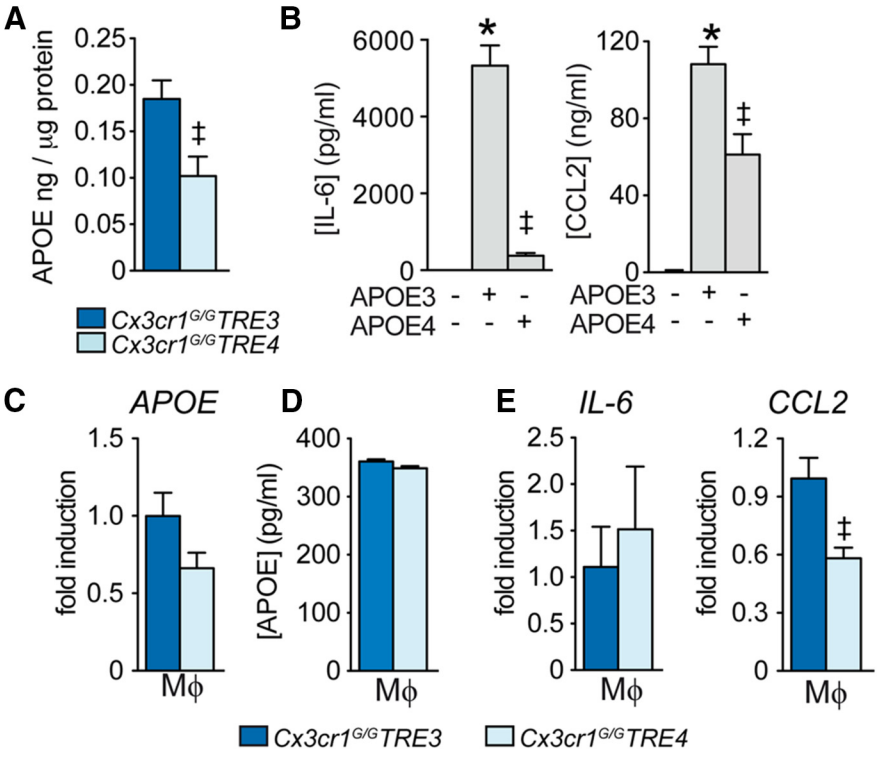

Figure 5. The *APOE4* allele decreases ocular APOE levels in *Cx3cr1*$^{GFP/GFP}$ mice and activates the IIRC inefficiently. ***A***, APOE ELISA of homogenates of PBS-perfused posterior segments of 12-month-old *Cx3cr1*$^{GFP/GFP}$*TRE3* mice and *Cx3cr1*$^{GFP/GFP}$*TRE4* mice (n = 8–11/group). *p = 0.0276 (Mann–Whitney t test). ***B***, Human IL-6 and CCL2 ELISA of supernatants from human monocytes incubated for 24 h in control medium, APOE3 (5 μg/ml), or APOE4 (5 μg/ml) (n = 6). $p < 0.0001$, IL-6 and CCL2 (one-way ANOVA/Bonferroni multiple-comparison tests). *$p < 0.05$, different from control (one-way ANOVA/Bonferroni multiple-comparison tests). ‡$p < 0.05$, different from APOE3 (one-way ANOVA/Bonferroni multiple-comparison tests). ***C***, ***D***, Quantitative RT-PCR of *ApoE* mRNA normalized with *S26* mRNA (***C***, n = 4) and APOE-ELISA of supernatants (***D***, n = 4) of peritoneal Mϕs from *TRE* mice cultured for 24 h. ***E***, Quantitative RT-PCR of Il-6 and Ccl2 mRNA normalized with *S26* mRNA (n = 5–10) of peritoneal Mϕs from *TRE* mice cultured for 24 h. ‡p = 0.0035, compared with CCL2 from Cx3cr1G/GTRE3 Mϕs (Mann–Whitney t test).

Discussion

We have previously shown that the lack of the tonic inhibitory CX3CL1/CX3CR1 signal, observed in *Cx3cr1*-deficient mice, is sufficient to induce pathogenic chronic subretinal MP accumulation due to increased CCL2-dependent monocyte recruitment and IL-6-dependent decrease of subretinal MP elimination (Combadière et al., 2007; Sennlaub et al., 2013; Hu et al., 2015; Levy et al., 2015). We showed that this accumulation is dependent on the overexpression of APOE in *Cx3cr1*-deficient MPs (Levy et al., 2015). As the APOE isoforms are associated with significant differences in APOE levels in humans (Mahley and Rall, 2000) and in humanized transgenic mice expressing APOE isoforms (Bales et al., 2009; Yu et al., 2013), we here evaluated the consequences of the three isoforms on chorioretinal homeostasis.

Our study shows that *TRE2* mice, carrying the AMD risk allele, develop age-, light-, and laser-induced subretinal MP accumulation associated with photoreceptor degeneration and excessive CNV. *TRE2* mice displayed increased tissue levels of APOE measured in whole retinal/choroidal protein extracts compared with *TRE3* and *TRE4* mice. This increase is likely due to reduced LDLR-dependent APOE2 uptake (Fryer et al., 2005) of APOE that is produced in the RPE, the inner retina and by subretinal MPs (Anderson et al., 2001; Levy et al., 2015). Similar to *CX3CR1*$^{GFP/GFP}$ mice and AMD patients (Levy et al., 2015), subretinal MPs in *TRE2* mice stained strongly positive for APOE. *In vitro*, we show that the *APOE2* allele is associated with increased APOE transcription and secretion in macrophages from *TRE2* mice, as previously shown for astrocytes (Yu et al., 2013). The APOE levels in and around subretinal MPs are therefore likely elevated because of increased APOE transcription and decreased LDLR-dependent clearance in the tissue. It is not yet clear to what extent APOE from nonmyeloid cells participates in the subretinal inflammation and whether extracellular or intracellular APOE within the MPs is the determining factor.

APOE is capable of activating the CD14/TLR2/TLR4-dependent IIRC and inducing IL-6, as previously shown for mouse macrophages (Smoak et al., 2010; Levy et al., 2015). We here confirm that APOE can induce inflammatory cytokines in a similar manner in human monocytes. Moreover, macrophages from *TRE2* mice that express significantly higher levels of APOE also transcribed higher levels of inflammatory cytokines, such as IL-6, CCL2, and IL-1β, in accordance with an APOE activation of the IIRC and similar to APOE-overexpressing *Cx3cr1*-deficient macrophages (Sennlaub et al., 2013; Hu et al., 2015; Levy et al., 2015). We previously showed that CCL2 (Sennlaub et al., 2013) and IL-6 (Levy et al., 2015) promote subretinal MP accumulation by increasing monocyte recruitment and decreasing MP clearance, respectively. Indeed, inhibition of the IIRC by a CD14-blocking antibody in laser-injured *TRE2* mice decreased subretinal MP accumulation and neovascularization. These results demonstrate that IIRC activation is significantly involved in the subretinal MP accumulation in *TRE2* mice *in vivo*. Together, *Cx3cr1*$^{GFP/GFP}$ mice and *TRE2* mice both overexpress APOE in mononuclear phagocytes, although for different reasons. In both mouse strains, the increased APOE is associated with IIRC activation, CCL2 and IL-6 induction, and pathogenic subretinal inflammation. Although these features do not mimic all the aspects of AMD (Drusen formation and RPE atrophy), they do model subretinal inflammation and associated photoreceptor degeneration, two hallmarks of AMD (Gupta et al., 2003). We previously demonstrated the importance of APOE in this process, as *APOE* deletion protected *Cx3cr1*$^{GFP/GFP}$ mice against the inflammation (Levy et al., 2015). Interestingly, increased levels of CCL2 and IL-6 are also observed in late AMD (Seddon et al., 2005; Jonas et al., 2010; Sennlaub et al., 2013; Chalam et al., 2014), where chronic MP accumulation is observed (Penfold et al., 2001; Gupta et al., 2003; Combadière et al., 2007; Sennlaub et al., 2013;

Levy et al., 2015). The observation that inhibition of subretinal MP accumulation in a variety of animal models represses CNV (Sakurai et al., 2003; Tsutsumi et al., 2003; Liu et al., 2013) and degeneration (Guo et al., 2012; Rutar et al., 2012; Suzuki et al., 2012; Kohno et al., 2013) strongly suggests that chronic subretinal inflammation partakes in AMD pathogenesis.

The chronic nonresolving inflammation in AMD is associated with an increase in APOE (Klaver et al., 1998; Anderson et al., 2001; Levy et al., 2015) similar to other inflammatory conditions (Rosenfeld et al., 1993). To evaluate whether a potential influence of the protective *APOE4* allele would become apparent in a situation of increased inflammation and APOE abundance, we crossed *TRE3* and *TRE4* mice to the APOE-overexpressing $Cx3cr1^{GFP/GFP}$ mice. In the inflammatory context of $Cx3cr1^{GFP/GFP}$ mice, the *APOE4* allele led to diminished APOE levels and the *APOE4* allele protected $Cx3cr1^{GFP/GFP}$ mice against harmful subretinal MP accumulation observed in *APOE3*-carrying $Cx3cr1^{GFP/GFP}$ mice. APOE4 is characterized, among others, by its decreased capacity to transport cholesterol compared with APOE3 (Heeren et al., 2004) and might thereby be less capable of modifying the cholesterol content of the lipid rafts and activating the IIRC (Smoak et al., 2010). Indeed, our results show that recombinant APOE4 induced less IL-6 and CCL2 compared with equimolar APOE3 concentrations in human monocytes *in vitro*. Similarly, macrophages from $Cx3cr1^{GFP/GFP}$*TRE4* mice transcribed less *CCL2* compared with macrophages from $Cx3cr1^{GFP/GFP}$*TRE3* mice, although their APOE expression was comparable. It is not clear why $Cx3cr1^{GFP/GFP}$*TRE4* macrophages did not differ from $Cx3cr1^{GFP/GFP}$*TRE3* macrophages in terms of *IL-6* transcription levels. Other unknown regulatory elements likely influence the transcription of the individual cytokines in macrophages, and the interplay of *Cx3cr1* deficiency and the human APOE3 and APOE4 isoform in the mouse macrophages might affect these pathways. We previously showed that CCL2 inhibition in $Cx3cr1^{GFP/GFP}$ mice significantly inhibited age-, laser-, and light-induced subretinal MP accumulation (Sennlaub et al., 2013) and diminished production of CCL2, as a result of reduced APOE concentrations and APOE4's impaired capacity to induce cytokines might explain the reduced inflammation and inhibition of degeneration and CNV observed in $Cx3cr1^{GFP/GFP}$ *TRE4* mice.

To our knowledge, this is the first study to describe a comprehensive pathomechanism of the involvement of APOE isoforms in AMD that is in accordance with the clinical observation of the *APOE2* allele being an AMD risk factor and the *APOE4* allele an AMD-protective genetic factor. One previous study demonstrated that *TRE4* mice on high-fat diet develop lipid accumulations in the Bruch's membrane, proposed as similar to early AMD (Malek et al., 2005), which are also observed in $ApoE^{-/-}$ mice (Ong et al., 2001). Although these observations might apply to early AMD, they are unlikely to play a role in late AMD in which increased APOE immunoreactivity is observed (Klaver et al., 1998; Anderson et al., 2001; Levy et al., 2015) and in which the *APOE4* allele plays a protective role (McKay et al., 2011). The involvement of increased reverse cholesterol transport in AMD might also be supported by the observation that APOA-I levels are elevated in the vitreous of AMD patients (Koss et al., 2014). Furthermore, a polymorphism of the ATP-binding cassette transporter 1 (associated with low high-density lipoprotein and therefore possibly impaired reverse cholesterol transport) has recently been shown to be protective against advanced AMD (Chen et al., 2010).

Our study also sheds an interesting light on the puzzling differences of the APOE isoform association with AMD (McKay et al., 2011) and AD (Mahley and Rall, 2000), two major age-related neurodegenerative diseases. In AD, the *APOE4* allele is associated with greater β-amyloid burden, possibly due to decreased APOE tissue concentrations and reduced efficacy in clearance of β-amyloid clearance via multiple pathways (Bales et al., 2009; Mahley et al., 2009). $Cx3cr1^{GFP/GFP}$ mice that express increased amounts of APOE in all MPs, including MCs (Levy et al., 2015), are protected against β-amyloid deposition in AD mouse models (Lee et al., 2010). In AMD, we show that excessive APOE expression associated with the AMD-risk *APOE2* allele leads to the induction of inflammatory cytokines that promote pathogenic subretinal inflammation (Figs. 1, 2, 3) (Levy et al., 2015), similar to *Cx3cr1*-deficient mice (Combadière et al., 2007; Sennlaub et al., 2013; Peng et al., 2014; Levy et al., 2015). On the other hand, we show that the *APOE4* allele is protective in the context of APOE overexpression of $Cx3cr1^{GFP/GFP}$ mice (Levy et al., 2015), due to decreased APOE tissue concentrations (Riddell et al., 2008; Bales et al., 2009; Sullivan et al., 2011) and its reduced capacity to induce inflammatory cytokines (Figs. 4, 5).

Together, our study shows that the *APOE2* allele leads to increased APOE expression, IIRC activation, and subretinal inflammation, whereas the *APOE4* allele diminishes IIRC activation and inflammation. Our study provides the rationale for the previously unexplained implication of the APOE genotype in AMD and opens avenues toward therapies inhibiting pathogenic chronic inflammation in late AMD.

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